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TITLE OF THE INVENTION
FIBRIN CELL SUPPORTS AND METHODS OF USE THEREOF

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FIBRIN CELL SUPPORTS AND METHODS OF USE THEREOF

FIELD OF THE INVENTION

The present invention relates to a fibrin cell support for cell cultures, containing a coagulated mixture of plasma proteins including fibrinogen and thrombin, its use in the preparation of keratinocyte cultures and their transport in the form of reconstituted epidermises, and their use for therapeutic purposes.

BACKGROUND OF THE INVENTION

The reconstitution of a living skin similar to the human skin from a few cells obtained from a biopsy, or of a simplified skin performing the physiological functions of a normal skin, is being studied extensively with the aim of replacing skin damaged by a serious disease (genetic, etc.) or by trauma such as major burns.

The skin is a complex organ composed of three juxtaposed tissues: the epidermis, 85% of which is constituted by keratinocytes, which form the impermeable horny layer that isolates the body from the outside environment; the dermis, which comprises cells, including fibroblasts, separated by a connective tissue composed mainly of collagen; and the hypoderma, which includes the cells dedicated to storing fats. Artificial reconstitution of such a complex organ thus poses numerous problems.

The first tissue to have been partially reconstituted *in vitro* was the dermis. (*See* Bell *et al.*, Proc. Natl. Acad. Sci. 76:1979-1274).

Starting with skin biopsies, fibroblasts have been successfully established in cultures, first in monolayers, then, after a number of passages, by dispersing these cells in culture medium containing collagen (extracted from rat's tail tendons), the latter forming a gel and permitting three-dimensional cultures. In such cultures, the fibroblasts interact with the collagen matrix, organizing and contracting it, as occurs in a normal dermis. This tissue, reconstituted *in vitro*, is known as an "equivalent dermis". After a few weeks' growth, the mechanical qualities of the equivalent dermis allow it to be used for grafting onto a patient or injured person. It does not

appear to be rejected by its host. However, this equivalent dermis is merely a temporary dressing as it cannot restore the skin's cutaneous barrier function.

Furthermore, a method and a culture medium enabling keratinocytes to be grown for long periods has been developed previously. (*See* Green et al. (1979) Proc. Natl. Aced. Sci. 76:5665). This method includes the step of inoculating the keratinocytes dispersed with trypsin on a pre-established monolayer of fibroblasts, in particular 3T3 cells, which have been lethally irradiated and which serve as a nutritive layer and as a matrix. An epidermal layer develops very rapidly to form a tissue having a thickness of 3 to 5 cells, and it can be grafted onto a patient and continue to differentiate *in situ*. This technique has been used to treat patients suffering from severe burns. (*See* Gallico *et al.* (1984) New England J. Med. 311:448).

Using the technique of Green *et al.*, it is possible to obtain, from a biopsy of two square centimeters, an epidermis of one square meter in the space of three weeks.

However, recovery of the reconstituted tissue in order to make a graft therefrom still poses a number of technical problems. It is necessary to detach the cells from the culture dish using an enzyme treatment without dissociating the cells. During this procedure, a retraction of the cell layer, and hence a loss of a certain percentage of the surface area of the graft, is observed. Moreover, once the reconstituted tissue has been detached, it has to be fastened to a support that enables it to be transported and grafted onto the patient. Typically, an adhesive-treated gauze dressing is generally used. These manipulations are both delicate and time consuming.

Thus, it would thus be highly beneficial to have at one's disposal novel fibrin cell supports that can be resorbed by the patient who has received the graft and that simplify the handling of the cells. In addition, to ensure their availability, such supports or their constituents should lend themselves to preparation and packaging in accordance with industrial processes.

SUMMARY OF THE INVENTION

In one aspect, the invention involves a fibrin cell support for cell cultures including calcic thrombin and fibrinogen, where the concentration of calcic thrombin is between about 0.5 U/ml and about 2.5 U/ml. Generally, the concentration of fibrinogen is between about 10 and about 250 mg/ml. In some embodiments of the present invention, the fibrin cell support includes

aprotinin, or other molecules including polypeptide growth factors, cytokines, enzymes, hormones, antibiotics, antimycotics, or a combination of two or more of these molecules. In other embodiments of the present invention, the fibrin cell support further includes one or more cells, *e.g.*, keratinocytes.

In another aspect, the present invention provides a method of preparing a fibrin cell support, including the steps of mixing equivalent volumes of a first solution comprising fibrinogen and a second solution comprising calcic thrombin; and distributing the mixture onto a surface, such that a fibrin cell support is formed on the surface. In certain embodiments of the present invention, this method further provides contacting with the fibrin cell support one or more cells, *e.g.*, keratinocytes.

In yet another aspect, the present invention provides a method of using a fibrin cell support, including the steps of contacting one or more keratinocytes with a fibrin cell support to form a skin replacement tissue, where the support includes calcic thrombin and fibrinogen; and recovering, transporting and applying the skin replacement tissue as a graft. The keratinocytes may be located on the surface of the fibrin cell support or integrated within the fibrin cell support.

In a further aspect, the present invention provides a method of using a skin replacement tissue, including the steps of contacting one or more keratinocytes with a solution comprising fibrinogen and calcic thrombin, to form a skin replacement tissue, and transporting the skin replacement tissue to a patient in need thereof.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel fibrin cell supports useful for culturing cells. These supports are formed by the coagulation of plasma proteins including fibrinogen in the presence of thrombin. This coagulation is chiefly due to the formation of a polymerized fibrin network, which imitates the formation of a blood clot. Thrombin converts fibrinogen to fibrin by enzymatic cleavage, and also converts protransglutaminase (factor XIII) to an active transglutaminase (factor XIIIa). Calcium accelerates the proteolytic activity of thrombin. To form a support suitable for the preparation of cell cultures, coagulation is carried out under conditions that are conducive to the formation of a film, and more particularly, in cell culture flasks or dishes.

One aspect of the present invention involves a fibrin cell support useful for culturing cells. This support is generated by combining a solution of a plasma protein such as fibrinogen with a solution of calcic thrombin such that a fibrin matrix forms, on which cells can be supported and cultured. The term "calcic thrombin" as used herein includes thrombin in the presence of calcium, such as a solution containing thrombin and any concentration of calcium.

Plasma proteins such as fibrinogen can be obtained from human plasma, (*e.g.*, obtained from blood donors) or can recombinant. The fibrinogen, if in solid form (such as freeze-dried or lyophilized), must be reconstituted, *e.g.*, in an isotonic solution. In some embodiments of the present invention, the isotonic solution is isotonic sodium chloride containing calcium chloride. The concentration of sodium chloride may be in the range of about 0.5% to about 5.0%, preferably in the range of about 1.0% to about 3.0%, and the concentration of calcium chloride may be in the range of about 0.5mM to about 5mM, preferably in the range of about 1mM to about 2mM. The isotonic solution may further comprise one or more protease inhibitors, *e.g.*, a polyvalent protease inhibitor such as aprotinin, provided in a concentration range of about 1000-5000 KIU/ml (kallikrein inhibitor units/ml), preferably about 3000 KIU/ml. Alternatively, such protease inhibitor(s) in solution may be added directly to the fibrinogen to reconstitute the protein. The concentration of fibrinogen is usually about 1-1000 mg/ml, preferably 10-250 mg/ml, more preferably 50-150 mg/ml. The fibrinogen solution may additionally contain other plasma proteins or polypeptides, such as fibronectin, Factor VIII and Factor XIII.

Likewise, thrombin may also be derived from natural sources or may be recombinant. Thrombin, if in solid form, can be reconstituted in an isotonic solution containing calcium, *e.g.*, 1.1% NaCl containing 1 mM calcium chloride. The concentration of the thrombin solution is usually about 0.1-10 U/ml, preferably 0.5-5.0 U/ml, even more preferably 1-3 U/ml and most preferably 2.5 U/ml. Units of thrombin refer to the activity standard as defined in the art. Thrombin may also be combined with fibrinogen in the absence of calcium. However, those skilled in the art will recognize that the presence of calcium accelerates the proteolytic activity of thrombin.

The fibrinogen solution and the thrombin solution are combined (usually in equal volumes) and are distributed to a vessel, such as a tissue culture dish, before clotting occurs. Once clotting occurs a fibrin cell support or matrix is formed. Alternatively, the two solutions may be injected into a vessel simultaneously using two syringes interconnected by a mixing coupling. Generally, the fibrin matrix formed by the combination of the calcic thrombin and the fibrinogen solutions will be transparent. The volume of the solution containing fibrinogen and thrombin used is dependent upon the thickness of the fibrin cell support desired. Typically, about 2.5mls of solution is used for every 100cm^2 of surface.

Those skilled in the art will recognize that other polypeptides or molecules (*e.g.*, growth factors or cytokines such as EGF, VEGF, PDGF, NGF, and TGF-β; healing agents; enzymes such as matrix-degrading enzymes and matrix-degrading enzyme inhibitors (*e.g.*, TIMPs); antibiotics and/or and antimycotics) may be added to the fibrinogen solution and/or the thrombin solution prior to, concomitant with or following the mixing of calcic thrombin and fibrinogen. The plasma transglutaminase factor XIIIa may be added to the fibrinogen solution, the thrombin solution, the mixture, or to the fibrin cell support in order to covalently crosslink the resulting fibrin cell support.

The fibrin cell support according to the invention is advantageous when preparing keratinocyte cultures, particularly human keratinocyte cultures. These cells can be either primary cultures derived from skin biopsies obtained from a patient that have undergone between 1 and 4 or more passages in 1/15 to 1/20 dilutions, or cells preserved in the form of banks in liquid nitrogen. Keratinocytes may be cultured in the presence of a feeder cell layer, such as a layer of lethally-irradiated human fibroblasts (*See* Limat et al., 1986 J Invest Dermatol. 1986 Oct;87(4):485-8).

In some embodiments of the present invention, keratinocytes are grown to confluence, trypsinized, suspended in an appropriate culture medium, and replated on the fibrin cell support. Keratinocytes may also be plated on the fibrin cell support at a subconfluent density and allowed to reach confluence in culture on the fibrin cell support. Keratinocytes may also be added to the mixture of thrombin and fibrinogen prior to coagulation, such that the keratinocytes are embedded within the fibrin cell support.

Cells amenable to use with the fibrin cell support of the invention include keratinocytes, but one of ordinary skill in the art will recognize that other cells, *e.g.*, endothelial cells, may be used instead of or in addition to keratinocytes. The cells of the present invention may be obtained from humans or other suitable mammals, and may be obtained from embryonic, neonatal or adult animals. The cells of the present invention may also include recombinant or genetically modified cells.

The use of the fibrin cell support according to the invention can be adapted in multiple ways. By way of non-limiting examples, the fibrin cell support may be used as follows.

According to one method of use, the fibrin cell support is prepared in the form of a film, by mixing its two constituents (calcic thrombin and fibrinogen) in a culture dish. A suspension of keratinocytes is then seeded on this film, in an appropriate culture medium. When the keratinocyte culture has become confluent or semi-confluent, it forms a replacement tissue that can be recovered directly as a graft, which can be detached using forceps and transported from the culture dish to the patient. It can be applied to the wound as is, without any need for a temporary support such as gauze. This method leads to a considerable saving in working time as well as a 100% recovery of the tissue grown.

According to another method of using the fibrin cell support according to the invention, the two constituents of the support are mixed with a keratinocyte suspension in such a way as to integrate the cells in the film that is subsequently formed. According to this method, the two constituents can be mixed with the keratinocyte cell suspension in a culture dish and then used as a graft, as described above. This method may also be carried out directly on a wound site on a patient, which has been prepared to receive a graft, by spraying a mixture of the fibrin cell support and the cells onto the wound using a vector gas (nitrogen) at a pressure of 2 to 2.5 bars.

According to yet another method of using the support according to the invention, the two constituents are mixed on a cell layer of keratinocytes that has been pre-established in a culture

dish. This is done in such a way that the cells become coated with the film that is formed. In this method the cells can be detached and transported in order to be applied to a wound as a graft.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1: Preparation of a fibrin cell support for cell cultures

A fibrin cell support for cell cultures is prepared by mixing a solution containing fibrinogen and a solution containing calcic thrombin.

Lyophilized fibrinogen is reconstituted with 5 ml of aprotinin (3,000 KIU/ml; kallikrein inhibitor units/ml) then combined with 5 ml of 2.2% NaCl containing 2mM calcium chloride. Lyophilized thrombin is diluted in 1.1% NaCl containing 1mM calcium chloride to a final concentration. The solubilized fibrinogen and solubilized thrombin are mixed in a 1:1 ratio and dispensed into a cell culture dish or flask (2.5 mls of the fibrinogen-thrombin mixture per 100 cm^2 of culture dish surface) to form a fibrin cell support. The fibrin cell support is then covered in cell culture medium.

Example 2: Preparing a keratinocyte culture on the fibrin cell support

Human keratinocytes originating from a skin biopsy are cultured in the presence of lethally-irradiated human fibroblasts. (*See* Green et al. (1979) Proc. Natl. Aced. Sci. 76:5665).

A layer of confluent keratinocytes is trypsinized, replaced in suspension in culture medium and seeded at subconfluent density (*e.g.*, in a 1/10 dilution) on a tissue culture dish covered with the fibrin cell support prepared as described in Example 1. The keratinocytes are then allowed to reach confluence, at which point the resulting keratinocyte graft can be used in therapeutic methods. The fibrin cell support of this invention stands up well to handling and does not retract at the time of detachment, which makes it possible to recover 100% of the surface area of the cell layer of the culture.

Example 3: Recovery of a pre-established cell layer using the fibrin cell support

Keratinocytes are inoculated according to Green's conventional method, in a Petri dish covered with a layer of lethally irradiated fibroblasts. (*See* Green et al. (1979) Proc. Natl. Aced. Sci. 76:5665). When keratinocytes are confluent and formed of several layers of cells, the culture medium is removed, an EDTA solution is added for 1 hour 30 minutes. This is followed by washing twice with PBS. The fibrin cell support prepared as described in Example 1 is then poured directly onto confluent keratinocytes.

Upon coagulation of the fibrin cell support, it can be detached mechanically and used as a graft, as demonstrated in Example 2.

Example 4: Incorporation of keratinocytes into the fibrin cell support

Keratinocytes can be embedded within the fibrin cell support by any of several methods. In a first method, a syringe of solubilized fibrinogen and a syringe of solubilized thrombin containing the keratinocytes in suspension are prepared. These keratinocytes may be taken from a fresh, trypsinized culture or from a bank of cells preserved in liquid nitrogen. The two syringes are interconnected by means of a mixing coupling and the resulting fibrin cell support containing the cells is sprayed onto a tissue culture dish (or onto a wound prepared to receive the graft). In this method, the cells are held within the fibrin cell support during its coagulation. The spraying can be carried out using a vector gas (*e.g.*, nitrogen at a pressure of 2 to 2.5 bars) or any other method known to those skilled in the art. This spraying does not damage the cells or denature the polypeptides, and the cell layer can be observed to reform in culture. These cells should thus multiply normally when the mixture is sprayed, in a very thin layer, directly onto a wound.

In a second method, the solubilized fibrinogen and solubilized thrombin are mixed in a 1:1 ratio, combined with a solution containing keratinocytes, and dispensed into a cell culture dish or flask (2.5 mls of the fibrinogen-thrombin mixture per 100 cm^2 of culture dish surface) to form a fibrin cell support containing keratinocytes.

OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

[redacted]

What is claimed is:

1. A fibrin cell support for skin grafts, comprising calcic thrombin and fibrinogen, wherein the concentration of calcic thrombin is between about 0.5 U/ml and about 2.5 U/ml.

2. The fibrin cell support according to claim 1, wherein the concentration of fibrinogen is between about 10 and about 250 mg/ml.

3. The fibrin cell support according to claim 1, wherein the concentration of calcic thrombin is between about 1.0 U/ml and about 1.5 U/ml.

4. The fibrin cell support according to claim 1, wherein the concentration of calcic thrombin is about 1.25 U/ml and the concentration of fibrinogen is between about 75 and about 150 mg/ml.

5. The fibrin cell support according to any one of claims 1 to 4, further comprising aprotinin, wherein the concentration of said aprotinin is about 3000 KIU/ml.

6. The fibrin cell support according to any one of claims 1 to 3, further comprising one or more molecules selected from the group consisting of a polypeptide growth factor, a cytokine, an enzyme, a hormone, an antibiotic, and an antimycotic, or a combination thereof.

7. The fibrin cell support according to any one of claims 1 to 4, further comprising one or more keratinocytes.

8. A method of preparing a fibrin cell support, comprising:
   a) mixing equivalent volumes of a first solution comprising fibrinogen and a second solution comprising calcic thrombin, wherein the concentration of calcic thrombin is between about 1.0 U/ml and about 1.5 U/ml; and

[redacted]

b) distributing said mixture onto a surface, such that a fibrin cell support is formed on said surface.

9. The method of claim 8, further comprising contacting said fibrin cell support with one or more keratinocytes.

10. The method of claim 8, further comprising contacting said mixture with one or more keratinocytes prior to distributing said mixture on said surface, thereby integrating said keratinocytes into said fibrin cell support.

11. The method of claim 8, wherein said surface is a tissue culture dish or flask.

12. A method of using a fibrin cell support, comprising:

a) contacting one or more keratinocytes with a fibrin cell support to form a skin replacement tissue, said support comprising calcic thrombin and fibrinogen, wherein the concentration of calcic thrombin is between about 1.0 U/ml and about 1.5 U/ml; and

b) recovering the skin replacement tissue;

c) transporting the skin replacement tissue; and

d) applying the skin replacement tissue as a graft.

13. The method according to claim 12, wherein said keratinocytes are on the surface of said fibrin cell support.

14. The method according to claim 12, wherein said keratinocytes are integrated within said fibrin cell support.

15. The method according to claim 12, wherein said keratinocytes are obtained after dispersion of a fresh cell layer.

16. The method according to claim 12, wherein said keratinocytes are obtained from a bank of cells preserved in liquid nitrogen.

17. A method of using a skin replacement tissue, comprising:

a) contacting one or more keratinocytes with a solution comprising fibrinogen and calcic thrombin, wherein the concentration of calcic thrombin is between about 1.0 U/ml and about 1.5 U/ml, to form a skin replacement tissue, and

b) transporting said skin replacement tissue to a patient in need thereof.

18. The method of claim 17, wherein said solution is added to a cell layer comprising keratinocytes in a culture dish.

19. The method of claim 17, wherein said keratinocytes are obtained from a bank of cells preserved in liquid nitrogen.

[redacted]

ABSTRACT

The present invention relates to a fibrin cell support for cell cultures formed by the mixture of plasma proteins including fibrinogen and thrombin. The fibrin cell support is preferably used for preparing a culture of keratinocytes, recovering the culture in the form of a reconstituted tissue, and transporting same. The reconstituted tissue is thus particularly suitable for use as a skin graft.

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